Exhibit D

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Increased muscle ubiquitin mRNA levels in gastric cancer patients

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Bossola, Maurizio, Maurizio Muscaritoli, Paola Costelli, Rocco Bellantone, Fabio Pacelli, Silvia Busquets, Josef Argilès, Francisco J. Lopez-Soriano, Ignazio M. Civello, Francesco M. Baccino, Filippo Rossi Fanelli, and Giovan Battista Doglietto. Increased muscle ubiquitin mRNA levels in gastric cancer patients. *Am J Physiol Regulatory Integrative Comp Physiol* 280: R1518–R1523, 2001.—The intramuscular ATP-dependent ubiquitin (Ub)-proteasome proteolytic system is hyperactivated in experimental cancer cachexia. The present study aimed at verifying whether the expression of the muscle Ub mRNA is altered in patients with cancer. Total muscle RNA was extracted using the guanidinium isothiocyanate/phenol/chloroform method from rectus abdominis biopsies obtained intraoperatively from 20 gastric cancer (GC) patients and 10 subjects with benign abdominal diseases (CON) undergoing surgery. Ub mRNA levels were measured by northern blot analysis. Serum soluble tumor necrosis factor receptor (sTNFR) was measured by ELISA. Ub mRNA levels (arbitrary units, means ± SD) were 2,345 ± 195 in GC and 1,162 ± 132 in CON ($P = 0.0005$). Ub mRNA levels directly correlated with disease stage ($r = 0.608$, $P = 0.005$), being 1,945 ± 786 in stages I and II, 2,480 ± 650 in stage III, and 3,799 ± 66 in stage IV. Ub mRNA and sTNFR did not correlate with age and nutritional parameters. This study confirms experimental data indicating an overexpression of muscle Ub mRNA in cancer cachexia. Lack of correlation with nutritional status suggests that Ub activation in human cancer is an early feature that precedes any clinical sign of cachexia.

cachexia; protein breakdown; proteolytic pathways

CACHEXIA IS A MULTIFACTORIAL syndrome characterized by anorexia, body weight loss (WL), and profound metabolic alterations (20), which accounts for one- to two-thirds of deaths in neoplastic patients (12). The progressive wasting significantly impairs both the quality of life and the response to antineoplastic therapies. However, despite the efforts in the last three decades, the management of cancer patients, based on nutritional interventions as well as on hormonal or pharmacological treatments (14), has met with little success.

Neoplastic patients frequently experience loss of both adipose tissue and skeletal muscle mass. Muscle wasting is generally accepted to result from an increase in protein breakdown with little or no change in protein synthesis (2), although the proteolytic pathways responsible for protein wasting have not been fully elucidated yet.

Skeletal muscle contains at least four main intracellular proteolytic systems that appear to serve distinct functions (17). Acidic proteases in lysosomes degrade endocytosed proteins and most membrane components. Intralysosomal protein digestion seems not relevant to the muscle catabolic response in cancer cachexia (CC), because the increased proteolysis in tumor-bearing rats is not affected by inhibition of lysosomal cathepsins (4, 33, 34). A second proteolytic system in muscles is the calcium-dependent one, which presently comprises at least three proteases (calpain I and II and the muscle-specific calpain p94) and the activity of which is regulated by calpastatin, a physiological inhibitor (7). The calcium-dependent proteolysis has been recently involved in a number of physiological and pathological processes such as repair of muscle damage and muscular dystrophy (7, 13, 26). These data, however, suggest a role for calpains in the modification of specific target proteins rather than in bulk protein degradation. A third degradation system, the role of which is not completely understood, neither involves lysosomes nor requires calcium or ATP (15, 17). Finally, the major cytosolic proteolytic pathway is a multienzymatic system that requires ATP and the polypeptide cofactor ubiquitin (Ub) (9). It involves an enzymatic cascade by which multiple Ub molecules are covalently attached

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to the protein substrate that is then degraded by the 26S proteasome complex. This pathway plays a primary role in the degradation of short-lived regulatory or abnormal proteins (9). Recently, it has been proposed that the increased muscle protein degradation occurring in several pathological states may result from an upregulation of the ATP-Ub-dependent pathway (30). In particular, clinical studies have confirmed the involvement of this proteolytic system in the skeletal muscle catabolism that characterizes severe wasting syndromes such as sepsis, trauma, and AIDS (23, 24, 27, 37).

Previous results from our laboratories have shown that overexpression of Ub mRNA closely parallels the enhancement of protein degradation in the skeletal muscle of rats bearing the Yoshida AH-130 ascites hepatoma (11, 24). Moreover, treatment of the AH-130 hosts with agents able to block the onset of tissue protein hypercatabolism, such as anti-tumor necrosis factor (TNF) antibodies or clenbuterol, also reduce muscle Ub mRNA levels (11, 21).

The aim of the present study has been to investigate whether human cancer, in which cachexia is clinically detectable relatively late in the course of the disease, also elicits perturbations of Ub expression in the skeletal muscle. Northern blotting analysis revealed that cancer patients overexpress Ub mRNA early in the course of the disease and that this elevation positively correlates with the disease stage.

METHODS

The study was approved by the local ethics committees. Twenty consecutive patients affected by gastric cancer admitted to the Istituto of Clinica Chirurgica of the Università Cattolica del Sacro Cuore of Rome and to the Dipartimento di Medicina Clinica of the Università La Sapienza, Rome, between January 1998 and January 1999, were included in the study protocol. Patients' characteristics are showed in Table 1. Diagnosis of gastric cancer was performed by endoscopic biopsy. Ten patients undergoing surgery for benign abdominal diseases served as a control group. Exclusion criteria for both groups were considered: acute or chronic renal failure (serum creatinine >1.2 mg/dl), liver failure, diabetes, metabolic acidosis, sepsis, AIDS, inflammatory bowel disease, autoimmune disorders, chronic heart failure, and hyperthyroidism.

Table 1. *Characteristics of the subjects studied*

	Controls (n = 10)	Gastric Cancer Patients (n = 20)
Age, yr	62.3 ± 14.5	61.8 ± 17.8
Sex (M:F)	6:4	11:9
Weight, kg	70.2 ± 6.8	67.9 ± 14.9
BMI	27.3 ± 4.3	25.3 ± 5.2
Weight loss, %	0.8 ± 0.4	5.6 ± 4.9*
Serum albumin, g/dl	4.1 ± 0.4	4.1 ± 0.5
Total lymphocyte count, number·counts^{-1}·min^{-1}·ml^{-1}	1,860 ± 850	1,692 ± 924
sTNFR, pg/ml	748 ± 40	1,045 ± 69†
Cortisol, pg/ml	169 ± 77	160 ± 80
FT3, pg/ml	2.34 ± 0.39	2.36 ± 0.46
FT4, pg/ml	12.7 ± 4.2	12.3 ± 3.9

Values are means ± SE; n, no. of cancer patients. M, male; F, female; BMI, body mass index; sTNFR, soluble tumor necrosis factor receptor. *P = 0.05 vs. controls; †P < 0.05 vs. controls. Weight loss is with respect to usual body wt.

Protocol. Written informed consent for the study procedures was obtained from the patients. All subjects were studied at 8:00 AM after overnight fasting. Blood samples for subsequent biochemical and hormonal analyses were obtained from an antecubital vein immediately before entering the operating room.

Nutritional assessment. The nutritional assessment included anthropometric [height, actual body weight, %WL, body mass index (BMI), usual body weight], immunological (total lymphocyte count), and biochemical (serum albumin) indexes.

Muscle biopsy. A biopsy specimen was obtained from the rectus abdominis muscle during the initial phase of the operation. The anterior sheet of the rectus abdominis muscle was opened with scissors after skin incision and dissection through the subcutaneous fat, and a muscle biopsy specimen weighing ~0.5 g was obtained. The biopsy specimen was divided into two portions that were immediately frozen in liquid nitrogen and then stored at −70°C until analysis. One portion of the biopsy was used for Northern blot analysis (see *RNA isolation and Northern blot analysis*). Small bleeding vessels were carefully controlled with ligatures and cautery after the muscle biopsy had been obtained, whereafter the operation continued in a routine fashion. No complications occurred from the biopsy procedure.

Serum hormones and cytokine. Serum fT_3, fT_4, and cortisol were determined by radioimmunoassay (Diagnostic System Laboratories, Webster, TX). Circulating levels of soluble TNF-α receptor (sTNFR) were determined by using an ELISA kit (Quantikine, R&D System).

RNA isolation and Northern blot analysis. Total RNA from rectus abdominis muscle was extracted using the guanidinium isothiocyanate/phenol/chloroform method as described by Chomczynski and Sacchi (8). RNA samples (40 μg/ml) were denaturated, subjected to 1.2% agarose gel electrophoresis, and transferred to Hybond H membranes (Amersham International, Buckinghamshire, UK). RNA was fixed to the membrane by ultraviolet illumination for 4 min.

Prehybridization was done in 50% formamide/5× sodium chloride-sodium citrate (SSC; 1× is 0.3 M NaCl, 65 mM sodium citrate)/5× Denhart's solution (1× Denhart's solution is 0.1% polyvinylpyrolidone, 0.1% Ficoll, 0.1% BSA)/20 mM sodium phosphate, pH 6.8/0.1% SDS μg/ml denaturated salmon sperm DNA overnight at 42°C. Membranes were hybridized with appropriate probes (10^6–10^7 counts·min^{-1}·ml^{-1}) at 42°C for 18 h. Nonspecifically bound probe was removed by successive washes in 2× SSC (15 min at 55°C, twice), 2× SSC + 0.1% SDS (30 min at 55°C), and 0.1× SSC + 0.1% SDS (15 min at 55°C, twice). Specific hybridization was then detected by autoradiography [for more details, see Llovera et al. (24)]. Radiolabeled probes were prepared by the random-priming method (Boehringer-Mannheim, Barcelona, Spain). The Ub probe used was a cDNA clone containing 12 bp of the second Ub coding sequence plus a complete third and fourth Ub coding sequence and 120 bp of the 3′-untranslated region of the chicken polyUb gene UBI (6). A probe for the 18S ribosomal subunit was used as a correction factor to quantitate Ub mRNA units. Filters were exposed to X Omat AR-5 films (Eastman Kodak, Rochester, NY) at −70°C for 2–4 days.

Data presentation and statistics. Data are presented as means ± SD. For each parameter, patients and controls were

compared by Student's *t*-test for unpaired data and Mann-Whitney *U* test, as appropriate. Coefficients of correlations were calculated by parametric and nonparametric regression analysis, as appropriate. A P value <0.05 was considered statistically significant.

RESULTS

Tables 1 and 2 show patients' and controls' characteristics. Cancer patients were divided into three subgroups according to the UICC classification (38) of tumor stage: group I-II including stages 1a, 1b, and 2; group III including stages 3a and 3b; and group IV including stage 4. Mean WL (%) with respect to usual body weight was significantly higher ($P = 0.005$) in cancer patients with respect to controls. When patients were stratified according to the severity of WL with respect to their usual body weight (mild: 0–5%, moderate: 6–10%, and severe: >10%), 8 of 20 had mild, 4 of 20 had moderate, and 3 had severe WL. No correlation was found between disease stage and WL. Serum fT_3 and fT_4 did not differ between the two groups (Table 1) and were unaffected by the nutritional status, whereas mean circulating levels of sTNFR were significantly higher ($P = 0.038$) in cancer patients than in controls (Table 1).

Northern blot analysis of the skeletal muscle revealed higher mRNA levels for Ub in gastric cancer patients than in control subjects (Fig. 1). Quantitation of the Ub mRNA levels (expressed in arbitrary units; means ± SD) showed a twofold increase in muscle from neoplastic patients with respect to controls (2,345 ± 195 vs. 1,162 ± 132, $P = 0.0005$). The levels of Ub mRNA did not correlate with age ($r = -0.15$; $P = 0.62$), percent WL ($r = 0.43$; $P = 0.11$), and total lymphocyte count ($r = 0.0357$; $P = 0.174$) as well as with serum cortisol ($r = 0.058$; $P = 0.88$), fT3 ($r = -0.195$; $P = 0.543$), sTNFR ($r = 0.066$; $P = 0.79$), serum albumin ($r = -0.054$; $P = 0.83$), and BMI ($r = -0.005$; $P = 0.98$). The levels of Ub mRNA and serum sTNFR were not influenced by the magnitude of WL (Table 3).

Levels of Ub mRNA (arbitrary units) were higher in stage IV (3,799 ± 66, $n = 2$) than in stages I and II (1,945 ± 786, $n = 10$; $P = 0.009$) and stage III (2,480 ± 650, $n = 8$; $P = 0.026$; Table 4). Spearman rank test revealed a direct correlation between Ub mRNA levels and disease stage ($r = 0.608$; $P = 0.005$).

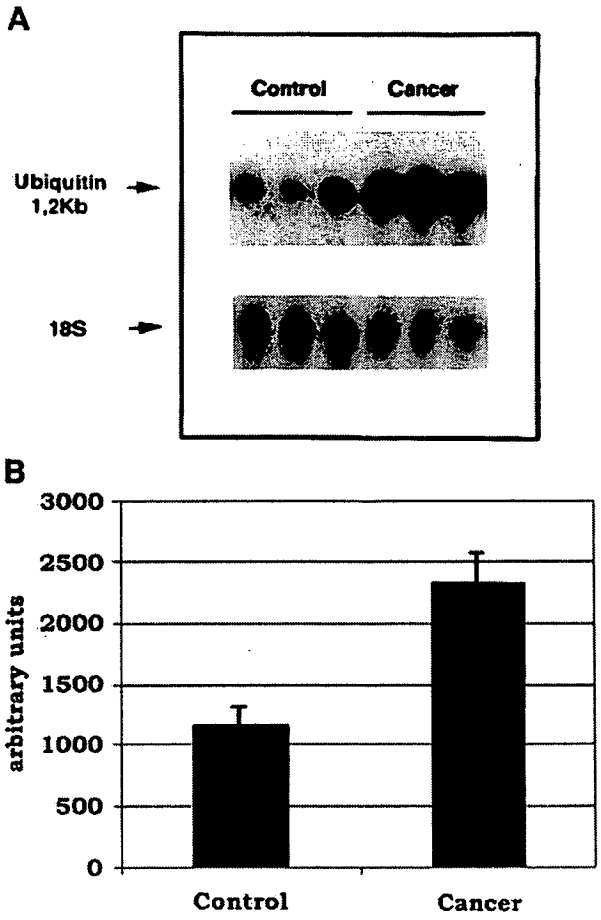

Fig. 1. Northern blot analysis of ubiquitin mRNA levels in rectus abdominis muscle biopsies from control and cancer patients. *A*: representative pattern of ubiquitin mRNA expression. Ribosomal 18S subunit was used as a correction factor for ubiquitin mRNA quantitation. *B*: scanning densitometric analysis in controls ($n = 10$) and cancer patients ($n = 20$). Significance of the differences: $P = 0.0005$ cancer patients vs. controls.

Table 2. *Clinical characteristics of the subjects included in the study*

	n
Controls	
Inguinal hernia	3
Cholelithiasis	3
Laparocele	4
Total	10
Gastric cancer	
Stage I–II	10
Stage III	8
Stage IV	2
Total	20

Values are nos. of patients.

DISCUSSION

Muscle wasting is one of the main features in CC and is mostly ascribed to enhanced protein catabolism (19, 28, 36). Changes in protein metabolism, not primarily due to malnutrition, can already be detected when the tumor is still very small (35) or undetectable (29). We

Table 3. *Levels of ubiquitin mRNA and serum sTNFR in cancer patients according to the severity of weight loss*

Weight Loss, %	Ubiquitin mRNA, Arbitrary Units	Serum sTNFR, pg/ml
0–5	2,338 ± 929	1,441 ± 1,155
6–10	2,581 ± 962	808 ± 129
>10	2,936 ± 756	969 ± 515

Values are means ± SE. Weight loss is with respect to body wt. Differences were not statistically significant.

Table 4. *Weight loss (with respect to usual body wt) and ubiquitin mRNA levels according to disease stage*

Stage	Mean Weight Loss, %	Ubiquitin mRNA Levels
I–II (n = 10)	3.9 ± 3.3	1,945 ± 786*
III (n = 8)	6.2 ± 3.4	2,481 ± 651†
IV (n = 2)	8.2 ± 3.2	3,799 ± 66‡

Values are means ± SE. Differences in weight loss between groups are not statistically significant: * vs. †, P = 0.126; † vs. ‡, P = 0.02; * vs. ‡, P = 0.009.

have previously shown that in an experimental model of CC, muscle protein hypercatabolism is associated with, and supported by, multiple alterations in the hormonal homeostasis and increased production of humoral mediators such as TNF-α and prostaglandin E_2 (10, 35). These alterations have been effectively antagonized with different pharmacological tools (10, 11, 34).

The precise mechanism by which skeletal muscle proteins are degraded is largely undetermined. The ATP-Ub-dependent proteolysis has been proposed to play a role in the turnover of long-lived proteins (16). In particular, perturbations of protein metabolism consequent to sepsis, burns, fasting, denervation, atrophy, or cancer have been associated with increased activity of this proteolytic pathway (4, 24, 33, 41). In experimental CC, the increased expression of Ub mRNA can be effectively suppressed by treatment with clenbuterol, an adrenergic β_2-agonist, or with anti-TNF antibodies (11, 21). However, the involvement of Ub-dependent proteolysis in human diseases has been addressed only in a few studies.

The present paper shows that Ub mRNA muscle levels are higher in gastric cancer patients than in controls. This observation is in line with the concept that in human cancer, lean body mass depletion may also result, at least in part, from an upregulation of the ATP-Ub-dependent proteolysis. Recent data from Williams et al. (40) demonstrating increased expression of genes pertaining to the Ub proteasome-proteolytic pathway in a small cohort of nonhomogeneous cancer patients are in agreement with the results of the present study.

The patients included in the present investigation were strictly selected for cancer site and histology (adenocarcinoma of the stomach), irrespective of the tumor stage and degree of WL to evaluate whether the level of Ub expression is related to these variables.

The expression of Ub mRNA indeed was influenced by the tumor stage, being higher in stages III and IV patients than in those with stage I or II cancer. Because Ub modulations have been shown to closely parallel protein breakdown rates in animal models and humans (5, 39), the positive correlation between Ub and tumor stage strongly suggests that muscle protein breakdown is progressively activated during the course of neoplastic disease. Accordingly, Ub mRNA levels might be proposed as a sensitive indicator of the muscle proteolytic state in neoplastic patients.

By contrast, the levels of Ub mRNA in the muscle of gastric cancer patients were consistently increased irrespective of the occurence of WL. This finding likely indicates that modulations of the muscle proteolytic machinery in cancer patients occur even before any clinical evidence of tissue wasting. Such observation may have pivotal clinical relevance because it would make a strong case for the systematic adoption of therapeutic interventions aimed at preserving lean body mass as soon as gastric cancer is diagnosed. Early nutritional and pharmacological strategies could in fact prevent the activation of protein breakdown thus antagonizing the onset of cachexia.

Humoral mediators released from tumor cells or generated in the host reaction to the tumor are currently believed to be involved in the pathogenesis of CC. In the present study, hormonal plasma levels were not significantly different between the two groups examined, whereas sTNFR concentrations were increased in cancer patients with respect to controls. The levels of sTNFR have been shown to closely correlate with those of TNF (32). The relationship between TNF circulating levels and tissue protein hypercatabolism has been demonstrated by several studies. TNF administration to experimental animals results in enhanced muscle protein breakdown (25), whereas treatment of AH-130-bearing rats with anti-TNF antibodies prevents muscle protein loss and reduces the increased expression of Ub mRNA (10, 21). Moreover, the presence of circulating TNF has been observed in children with acute lymphoblastic leukemia and in other cancer patients (1, 3, 22, 31). The increase of sTNFR levels observed in gastric cancer patients suggests the occurrence of perturbations in the cytokine network, which likely concur in driving the metabolic balance toward catabolism. In the present study, however, we did not observe any correlation between muscle Ub mRNA and serum sTNFR. This observation suggests that in addition to TNF, other cytokines such as interferon-γ and interleukin-1 (23) and humoral factors could be implicated in the activation of Ub proteasome-mediated proteolysis in this type of cancer.

Taken together, these results further corroborate the hypothesis that the metabolic and humoral alterations previously described in different experimental models of cachexia are present also in human cancer. The lack of correlation between either Ub expression or sTNFR levels with body weight or BMI suggests that these alterations occur early in the course of the disease when cachexia is not yet clinically relevant. The positive correlation between Ub mRNA levels and tumor stage suggests that severe muscle depletion is the result of a progressive activation of protein breakdown. Keeping this in mind, the importance of early detection of protein metabolism perturbations in cancer patients appears quite compelling.

Perspectives

Loss of lean body mass is a common feature in several wasting diseases and significantly contributes to impair the patient's outcome. CC is characterized by a reduction in the host's food intake and by enhanced protein degradation at the muscular level. The treat-

ment of CC has been challenging clinicians and basic scientists for decades, generating frustrations, hopes, but overall little improvement. At present, no effective pharmacological or nutritional approach to human CC has been devised that can significantly counterbalance the catabolic stimuli evoked by the presence of a cancer. During the recent years, animal studies and molecular biology have greatly contributed to gain insights into the mechanism regulating muscle protein degradation, suggesting that the ATP-dependent Ub proteasome-proteolytic pathway would play a major role in muscle wasting in a variety of clinical conditions including CC. The demonstration that in human cancer Ub mRNA levels are increased, even in the early phases of neoplastic disease, is of great importance on both a physiological and a therapeutic ground. In fact, it represents a further significant advancement to clarify the role played by this proteolytic system in a clinical setting characterized by accelerated muscle protein breakdown such as cancer. Moreover, it supports the thinking that a rational approach to cancer patients should try to pharmacologically counteract the deleterious effects deriving by the upregulation of this pathway early after cancer diagnosis. This is based on the observation that perturbations of this pathway in gastric cancer patients are already present long before the clinical appearance of body wasting and cachexia.

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